Neuroscience 220 (2012) 228–236

CO-REGULATION OF *SURVIVAL OF MOTOR NEURON* AND BCL-XL EXPRESSION: IMPLICATIONS FOR NEUROPROTECTION IN SPINAL MUSCULAR ATROPHY

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Abstract—Spinal muscular atrophy (SMA), a fatal genetic motor disorder of infants, is caused by diminished full-length *survival of motor neuron* (SMN) protein levels. Normally involved in small nuclear ribonucleoprotein (snRNP) assembly and pre-mRNA splicing, recent studies suggest that SMN plays a critical role in regulating apoptosis. Interestingly, the anti-apoptotic Bcl-x isoform, Bcl-xL, is reduced in SMA. In a related finding, Sam68, an RNA-binding protein, was found to modulate splicing of SMN and Bcl-xL transcripts, promoting SMNΔ7 and pro-apoptotic Bcl-xS transcripts. Here we demonstrate that Bcl-xL expression increases SMN protein by ~2-fold in SH-SY5Y cells. Conversely, SMN expression increases Bcl-xL protein levels by ~6-fold in SH-SY5Y cells, and ~2.5-fold in the brains of transgenic mice over-expressing SMN (PrP-SMN). Moreover, Sam68 protein levels were markedly reduced following SMN and Bcl-xL expression in SH-SY5Y cells, suggesting a feedback mechanism co-regulating levels of both proteins. We also found that exogenous SMN expression increased full-length SMN transcripts, possibly by promoting exon 7 inclusion. Finally, co-expression of SMN and Bcl-xL produced an additive anti-apoptotic effect following PI3-kinase inhibition in SH-SY5Y cells. Our findings implicate Bcl-xL as another potential target in SMA therapeutics, and indicate that therapeutic increases in SMN may arise from modest increases in total SMN. **© 2012 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: SMN, Bcl-xL, Sam68, PrP-SMN, additive, co-expression.

INTRODUCTION

Spinal muscular atrophy (SMA) is an often fatal autosomal recessive genetic disease typified by the degeneration of anterior horn cells of the spinal cord, muscle weakness and atrophy. With an incidence of one in 6,000 live births, SMA is the most common genetic cause of infant mortality. Clinically characterized by profound muscle weakness, hypotonia and trunk paralysis, SMA is classified into 3 main subtypes (I–III) based on disease severity and age of onset (Munsat and Davies, 1992).

SMA is a monogenic disorder whereby the *survival of motor neuron* (*SMN1*) gene is mutated or absent in more than 95% of cases (Lefebvre et al., 1995). During human evolution, a 500 kb inverted duplication of the *SMN1* locus has given rise to a second copy of the gene, termed *SMN2*. The *SMN2* gene is essentially identical to *SMN1* except for a single translationally silent nucleotide change (C→T) at position 6 of exon 7, which causes exclusion of exon 7 in SMN2 transcripts (Lorson et al., 1999; Monani et al., 1999). Consequently, only about 10% of SMN2 protein is functional, while the other 90% is truncated and unstable. The occurrence of multiple *SMN2* gene copies in patient groups correlates with increased full-length SMN transcripts, greater amounts of functional protein, and reduced disease severity (Lefebvre et al., 1997; Feldkotter et al., 2002).

The SMN protein is highly expressed during embryonic development and ubiquitously expressed within the cytoplasm and nucleus of cells (La Bella et al., 1998). SMN plays a role in the assembly of small nuclear ribonucleoproteins (snRNPs), a component essential for pre-mRNA splicing (Liu et al., 1997; Pellizzoni et al., 1999). Among other functions, SMN also interacts with key regulators of cell survival, such as Bcl-2, ZPR1 and p53 (Iwahashi et al., 1997; Gangwani et al., 2001; Young et al., 2002), and appears to possess an important anti-apoptotic function (Kerr et al., 2000; Vyas et al., 2002; Anderton et al., 2011).

Curiously, like SMN, expression of the anti-apoptotic Bcl-2 family member, Bcl-xL, is also reduced in SMA patients and SMA mouse models (Soler-Botija et al., 2003; Tsai et al., 2008). Bcl-xL is highly expressed in

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Abbreviations: DMEM, Dulbecco's modified eagle medium; GFP, green fluorescent protein; PBS, Phosphate buffered saline; PCR, Polymerase chain reaction; PI3-kinase, phosphatidylinositol-3 kinase; PrP-SMN, transgenic mouse over-expressing SMN via the prion promoter; SMA, spinal muscular atrophy; SMN, *survival of motor neuron protein*.

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http://dx.doi.org/10.1016/j.neuroscience.2012.06.042

the CNS and regulates cell survival by inhibiting the pro-apoptotic Bax and Bak proteins (Merry and Korsmeyer, 1997). Importantly, Bcl-xL expression rescues SMA-like motor defects (Garcera et al., 2011) and increases the life-span of transgenic type III SMA mice (Tsai et al., 2008). The co-occurrence of reduced Bcl-xL and SMN in SMA suggests co-regulated expression of these two proteins. Interestingly, phosphorylation of the RNA-binding protein, Sam68, promotes exon 7 inclusion in SMN2 transcripts and splicing of the pro-survival Bcl-xL transcript (Paronetto et al., 2007; Pedrotti et al., 2010).

We used recombinant adenoviral vectors and transgenic mice to investigate the potentially important relationship between SMN and Bcl-xL expression. Expression of SMN strongly increased Bcl-xL expression in SH-SY5Y cells, and in PrP-SMN mouse brain tissue. Remarkably, expression of both SMN and Bcl-xL reduced total Sam68 levels, supporting the notion that SMN positively regulates its own expression, possibly by modulating Sam68 levels. Finally, we demonstrate that co-expression of Bcl-xL and SMN has an additive neuroprotective effect against phosphatidylinositol-3 kinase (PI3-kinase) inhibition-induced apoptosis, thereby identifying Bcl-xL as a valid therapeutic target in SMA treatment.

EXPERIMENTAL PROCEDURE

SH-SY5Y and type 1 SMA patient fibroblast cell maintenance

SH-SY5Y neuroblastoma cells and SMA type I patient fibroblasts (GMO3813; Coriell Institute, Camden, NJ, USA) were maintained in Dulbecco's modified eagle medium (DMEM)-containing penicillin (20 units/ml), streptomycin (20 mg/ml) and fetal calf serum (FCS; 5–10%; heat-inactivated), and incubated at 37 °C (5% CO_2). The neuronal differentiation of SH-SY5Y cells by exposure to retinoic acid (15 μM; 5 days) has previously been described (Anderton et al., 2011).

Adenoviral vectors and transduction of cells in culture

Recombinant adenoviruses were prepared according to the method of He et al. (1998), with some modifications (Boulos et al., 2006). Adenoviral vectors expressing Bcl-xL (AdBcl-xL), SMN (AdSMN) and a control vector (AdEmpty) have been described previously (Boulos et al., 2006; Anderton et al., 2011). All adenoviral vectors express a green fluorescent protein (GFP) gene reporter. Briefly, adenovirus was diluted in serum-free DMEM media for use at an appropriate multiplicity of infection (MOI) (50–300) prior to addition to cells in cultures. To ensure uniform transduction of each adenoviral vector, cultures were routinely assessed for GFP reporter expression using epi-fluorescent imaging (Olympus IX70; Olympus DP70 digital camera). Cultures were used for experiments 72 h after adenoviral transduction.

Transgenic mice

Transgenic PrP-SMN mice (line 92; (Gavrilina et al., 2008)) were obtained from Prof. Kevin Talbot (University of Oxford) and maintained on an FVB/N background. Non-transgenic FVB/N littermates provided wild-type (WT) controls. Mice were killed at 2 months of age by lethal injection (sodium pentobarbitone, 100 mg/kg, IP). Whole brain and lumbar spinal cord were dissected out and snap-frozen. These experiments were approved by the Howard Florey Institute Animal Ethics Committee (permit number 10-024).

Protein extraction and Western blotting

Protein was extracted from mouse tissue or cultured cells using RIPA lysis buffer and homogenization. Western blotting has been described previously (Anderton et al., 2011). Briefly, membranes were blocked in PBS-Tween 20 (0.1%)-containing ovalbumin (1 mg/ml) for 1 h and incubated in α-fodrin (1:1000; MP Biomedicals, Solon, OH, USA), Bcl-x (1:3000: BD Biosciences, San Jose, CA, USA), β-tubulin (1:10000; Santa Cruz, Santa Cruz, CA, USA), Sam68 (1:5000; Santa Cruz), or SMN (1:3000; Santa Cruz) primary antibodies diluted in PBS-T (0.1%) plus ovalbumin (1 mg/ml). Proteins were detected using a HRP-complexed secondary antibody (1:15000–1:35000 donkey anti-rabbit/sheep anti-mouse; GE Healthcare, Piscataway, NJ, USA), and visualized using ECL plus detection reagent (GE Healthcare). Quantification and band densitometry of Western blots was undertaken using ImageJ (NIH) software.

Quantitative real-time-PCR

Total RNA was harvested using TRIzol® (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Five hundred nanograms of total RNA was DNase treated (TURBO DNA-free; Applied Biosystems, Foster City, CA, USA) prior to 10 μl being used for cDNA synthesis using SuperScript III RT (Invitrogen) primed with random hexamers (Invitrogen) at 100 ng/μl, according to the manufacturer's instructions. Quantitative real-time RT-PCR was performed using an ABI Prism 7900HT sequence detection system (Applied Biosystems). Amplification of specific PCR products was performed in triplicates in a total reaction volume of 15 μL-containing 2 μL cDNA template, forward and reverse primers (300 nM), and 2× Fast SYBR® master mix reagents (Invitrogen). Primers used for SMN amplification (forward; 5′ GTC CAG ATT CTC TTG ATG ATG C 3′ and reverse; 5′ CTC TAT GCC AGC ATT TCT CCT TA 3′) and GAPDH amplification (forward; 5′ ACA GTC AGC CGC ATC TTC TT 3′ and reverse; 5′ ACG ACC AAA TCC GTT GAC TC 3′). Amplifications were performed at 95 °C for 6 min and for 40 cycles of 20 s at 95 °C, 20 s at 60 °C and 30 s at 72 °C. For quantitative PCR analysis, differences in gene expression were assessed relative to the levels of an internal control (Pfaffl, 2001).

One step RT-PCR

One hundred nanograms of total RNA was used in reaction with SuperScript III One Step RT-PCR with Platinum Taq (Invitrogen) as per manufacturer's instructions. The SMN transcript was amplified across exons 4–8 using PCR primers (Forward: 5′ AGG TCT CCT GGA AAT AAA TCA G 3′ and Reverse: 5′ TGG TGT CAT TTA GTG CTG CTC T 3′). The amplification protocol followed 55 °C for 30 min, 94 °C for 2 min and 28 cycles of 94 °C for 40 s, 56 °C for 1 min and 68 °C for 1 min. PCR products were fractionated on a 2% agarose gel.

PI3-kinase inhibition apoptotic model

Assessment of the anti-apoptotic function of SMN using the PI3-kinase inhibitor, LY294002 (Sigma, St. Louis, MO, USA), has previously been described (Anderton et al., 2011). Briefly, LY294002 was diluted in serum-free DMEM to a final concentration of 25 μM prior to addition to differentiated SH-SY5Y cells.

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